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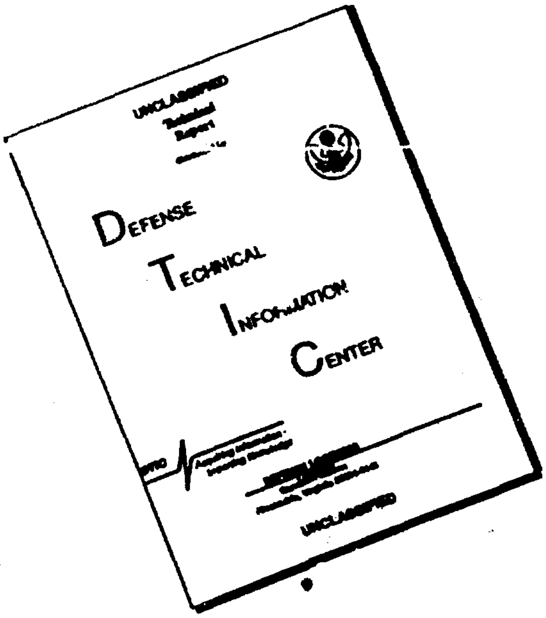

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DETERMINATION OF MICROQUANTITIES OF CYSTEINE AND GLUTATHIONE BY THE IODIDE-PERSULFATE REACTION

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Sulfhydryl groups play a large part in biological metabolism processes. There are various methods of determining them quantitatively, the basis of which is the high reactivity of the sulfhydryl groups.

Methods based on the oxidation reactions of thiols were widely used, owing to their sensitivity. But the oxidation reaction is not very specific; imidazole, hydroxyl and other groups are oxidized along with the sulfhydryl groups. Oxidation of the sulfhydryl groups does not always stop at the disulfide bond formation stage. The degree of oxidation of the sulfhydryl groups can change, depending on the reaction conditions (pH of the solution, concentration of the reagents, presence of metal ions, temperature), which leads to divergence from the stoichiometric ratios.

Among the oxidizing agents employed for determining the sulfhydryl groups iodine was widely used. Both direct titration with iodine and back titration of an excess of iodine with hyposulfite are used for the determination of thiols. The degree of oxidation of the sulfhydryl groups by iodine also depends on the conditions under which the reaction takes place. Thus, upon oxidation of cysteine to the disulfide bond an acid medium, low temperature, and an excess of iodide are necessary for the suppression of cysteic acid formation. The oxidation of cysteine to cysteic acid requires six equivalents of iodine. With an insufficient amount of iodine the intermediate products sulfinic acid and sulfonic acid are formed. The reaction between potassium iodide and potassium iodate was proposed for obtaining a standard concentration of iodine in the solution (1).

Many variants exist for the iodometric titration of sulfhydryl groups, but they are all unsuitable for determining microquantities of these groups.

In recent years investigations in which catalysis and kinetic indices are used for analytical purposes have been appearing with ever-increasing frequency. The determination of the initial reaction rate, calculation of the amount of any of the reaction products after specific time intervals, measurement of the time during which the substance reacted, i. e. the duration of the reaction — all these indices can also serve for the determination of microquantities of sulfhydryl groups.

It was ascertained by the authors of (2, 6, 7) that the reaction between sodium azide and iodine is catalyzed by divalent sulfur compounds, whereupon the rate of this reaction depends on the amount of the catalyst. Kur--wa (3) worked out a method of determining microquantities of cysteine and cystine on the basis of the different rates at which these substances catalyze the iodine azide reaction. Pardue and Shepherd (4) worked out a micromethod of determining cystine which was based on continuous potentiometric measurement of the decreasing concentration of iodine in the first seconds of the reaction. The rate of reduction of the iodine in the initial stage is a function of the cystine concentration.

In 1964 we developed a method of quantitative analysis of protein by the catalytic action of the copper of a biuret complex on the oxidation reaction of potassium iodide with potassium persulfate (5). The amount of protein is judged by the length of time in which the iodine being liberated combined with a definite amount of sodium hyposulfite present in the reaction mixture. The duration of the reaction depends not only on the amount of catalyst, but also on the hyposulfite concentration. Thus the carrying out of the reaction of oxidizing iodide with persulfate without a catalyst reveals a way to determine the concentration of the hyposulfite or other reducing agent. Compounds with sulfhydryl groups can be reducing agents of that type. The free iodine liberated upon reaction oxidizes the SH groups in the solution.

The reaction can be represented schematically in this form:

$$2\,KI + K_2S_2O_8 = 2\,K_2SO_4 + I_2$$

$$I_2 + 2\,RSH = RSSR + 2\,HI$$

After oxidation of all the sulfhydryl groups in the solution free iodine appears, which can be revealed by the starch-iodine reaction. The time from the start of the oxidation reaction of the sulfhydryl groups to the moment the blue coloration appears depends on the concentration of SH groups. The less the quantity of SH groups in the solution, the quicker the blue coloration begins to show. In order to determine microquantities of SH groups it was necessary to select conditions at which the oxidation reaction would proceed at a comparatively low rate. For this purpose the effect of such factors as the pH of the medium, the temperature, and the ratio of the KI and $K_2S_2O_8$ concentrations on the reaction rate was studied.

The effect of the pH on the reaction rate in the presence of 44 micrograms of cysteine is shown in Fig. 1. In the alkaline and acid zones the reaction rate is markedly slowed down. But the alkaline zone is not suitable for the method worked out, owing to auto-oxidation of the SH groups under these conditions. Thus it was ascertained that the pH range below 2 is the most suitable for the analysis of small concentrations of sulfhydryl groups.

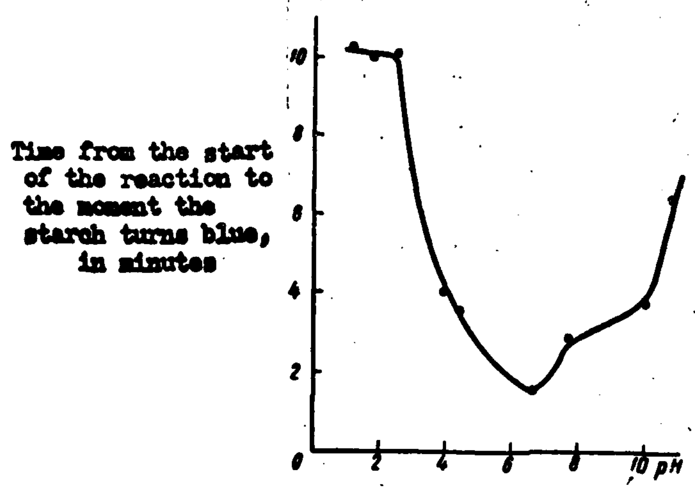


Fig. 1. Effect of pH on the Reaction Time of Oxidation of the Sulfhydryl Groups of Cysteine

The study of the effect of different concentrations of KI and $K_2S_2O_8$ on their reaction rate showed that a low rate with a clear-cut point at which the blue color appears can be obtained at concentrations of 0.01 M for KI and 0.004 M for $K_2S_2O_8$.

The determination of cysteine was carried out as follows: to 2.5 ml of the solution being investigated were added 0.5 ml HCl (1 N), 0.5 ml starch (one percent), 0.5 ml KI (0.1 M) and one ml $K_2S_2O_8$ (0.02 M) (potassium persulfate is added last, since it also causes oxidation of the sulfhydryl groups). The time from the moment of adding the persulfate to when the starch begins to turn blue was recorded by stopwatch. The reaction was carried out in a thermostat at 25 ± 0.02°. In Fig. 2 is shown the relation between the cysteine concentration and the time interval from the start of the reaction to the appearance of the blue coloration. On the ordinate is plotted the difference between the reaction time of the test sample and that of the control. Water taken in place of the test solution served as the control. The more cysteine there is, the more iodine is used up in oxidizing it, and the

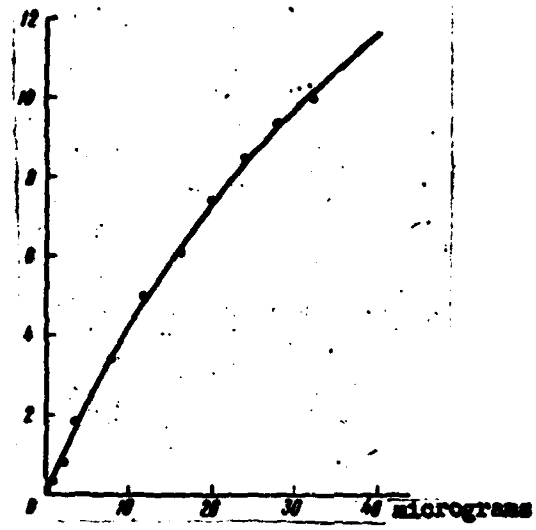


Fig. 2. Relationship Between the Cysteine Concentration and the Reaction Time of the Oxidation of its Sulfhydryl Groups

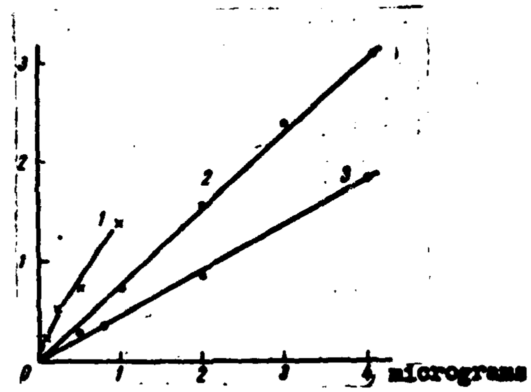
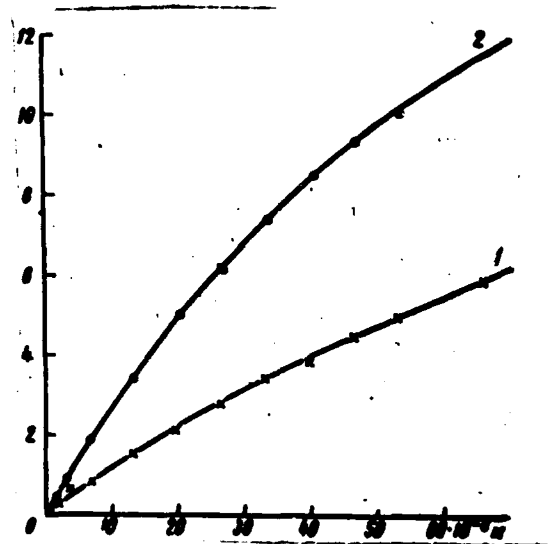


Fig. 3. Relationship Between the Cysteine Concentration and the Reaction Time of the Oxidation of its Sulfhydryl Groups at Different Temperatures
1 - 10°; 2 - 15°; 3 - 25°

later the starch-iodine reaction starts. Upon determination of cysteine within the 0.5 to 1000 microgram concentration range the time interval correspondingly varied from 0.5 seconds to 50 minutes. The error of the method is about 2.5 percent, but with a decrease in cysteine concentration the relative error increased. In order to increase the limit of sensitivity of the method it is necessary to decrease the reaction rate. It is convenient to do this by lowering the temperature. In Fig. 5 is represented the relation of the sensitivity of the cysteine determination to the temperature. At 25° 0.8 micrograms can be determined at a reaction time of 22 seconds; at 15° — 0.5 micrograms at a reaction time of 18 seconds; at 10° — 0.2 micrograms in 24 seconds.

Determination of glutathione under the same conditions showed that the reaction time in this case is considerably less than in the determination of cysteine (Fig. 4), i. e. the sensitivity of the reaction was insufficient. In order to increase it, it was necessary to change the reaction conditions. This was achieved by decreasing the concentration of the reagents — KI to 0.005 M and $K_2S_2O_8$ to 0.05 M.

Time from the start of the reaction to the moment the starch turns blue, in minutes



Molarity of the thiols in the reaction mixture

Fig. 4. Relationship Between the Concentration of Cysteine and Glutathione and the Reaction Time of Oxidation of Their Sulfhydryl Groups

1 - glutathione; 2 - cysteine

To 3 ml glutathione solution were added 0.5 ml HCl (1 N), 0.5 ml starch (one percent), 0.25 ml KI (0.1 M) and 0.75 ml $K_2S_2O_8$ (0.02 M).

The reaction was carried out at 25 ± 0.02° and at 15°. At 25° one microgram of glutathione can be determined at a reaction time of 17 seconds, and at 15° — 0.5 micrograms at a reaction time of 25 seconds (Fig. 5).

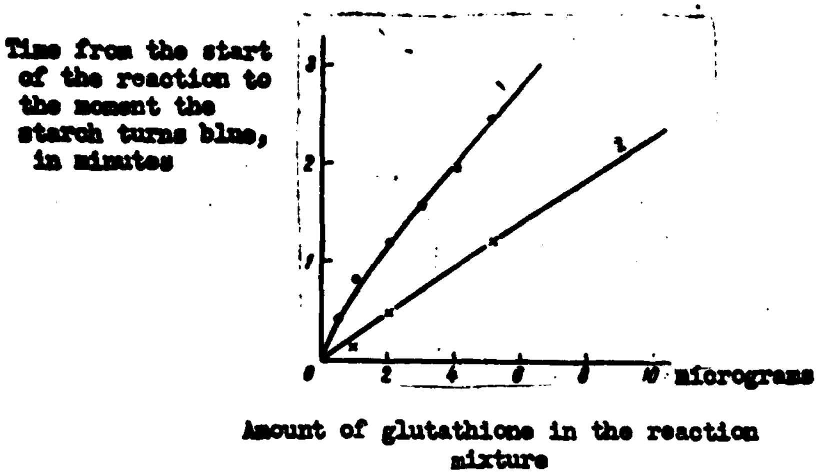


Fig. 5. Relationship Between the Glutathione Concentration and the Reaction Time of Oxidation of its Sulfhydryl Groups at Different Temperatures

1 - 15°; 2 - 25°

To explain the effect of other amino acids on the determination of the sulfhydryl groups a series of experiments was set up, in which the rates of reaction of potassium iodide with potassium persulfate in the presence of a number of amino acids were determined. Here it turned out that cystine, serine, valine, glycine, hydroxyproline, lysine, arginine, phenylalanine, tyrosine, tryptophane, asparagine and glutamic acid in amounts of up to 500 micrograms do not affect the reaction rate and do not interfere with the determination of cysteine and glutathione. Histidine slightly inhibits the reaction, but methionine accelerates it. Sulfides and sulfites, competing for the iodine being liberated, interfere with the determination of the sulfhydryl groups. Copper and iron ions catalyze the reaction; if they are present in small amounts, addition of the complexone suppresses their catalytic activity.

CONCLUSIONS

A micromethod of determining thiols of low molecular weight is proposed.

The method is based on measurement of the reaction time of the oxidation of the sulfhydryl groups by the iodine liberated upon the reaction of potassium iodide with potassium persulfate. Minimum amounts of the substance which can be determined are 0.8 micrograms for cysteine and one microgram for glutathione.

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